An agonist-induced switch in G protein coupling of the gonadotropin-releasing hormone receptor regulates pulsatile neuropeptide secretion

Lazar Z. Krsmanovic, Nadia Mores*, Carlos E. Navarro, Krishan K. Arora, and Kevin J. Catt†

Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-4510

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The pulsatile secretion of gonadotropin-releasing hormone (GnRH) from normal and immortalized hypothalamic GnRH neurons is highly calcium-dependent and is stimulated by cAMP. It is also influenced by agonist activation of the endogenous GnRH receptor (GnRH-R), which couples to $G_{q/11}$ as indicated by release of membrane-bound $\alpha_{q/11}$ subunits and increased inositol phosphate/Ca^{2+} signaling. Conversely, GnRH antagonists increase membrane-associated $\alpha_{q/11}$ subunits and abolish pulsatile GnRH secretion. GnRH also stimulates cAMP production but at high concentrations has a pertussis toxin-sensitive inhibitory effect, indicative of receptor coupling to G_i. Coupling of the agonist-activated GnRH-R to both G_s and G_i proteins was demonstrated by the ability of nanomolar GnRH concentrations to reduce membrane-associated α_s and α_{i3} levels and of higher concentrations to diminish α_{i3} levels. Conversely, α_{i3} was increased during GnRH antagonist and pertussis toxin treatment, with concomitant loss of pulsatile GnRH secretion. In cholera toxin-treated GnRH neurons, decreases in α_s immunoreactivity and increases in cAMP production paralleled the responses to nanomolar GnRH concentrations. Treatment with cholera toxin and 8-bromo-cAMP amplified episodic GnRH pulses but did not affect their frequency. These findings suggest that an agonist concentration-dependent switch in coupling of the GnRH-R between specific G proteins modulates neuronal Ca^{2+} signaling via G_s-cAMP stimulatory and G_i-cAMP inhibitory mechanisms. Activation of G_i may also inhibit GnRH neuronal function and episodic secretion by regulating membrane ion currents. This autocrine mechanism could serve as a timer to determine the frequency of pulsatile GnRH release by regulating Ca^{2+}- and cAMP-dependent signaling and GnRH neuronal firing.

The neuroendocrine control of reproductive function is expressed through the episodic secretion of gonadotropic hormones from the anterior pituitary gland in response to pulsatile stimulation by gonadotropin-releasing hormone (GnRH) produced in a network of peptidergic neurons in the hypothalamus. The underlying mechanism of the GnRH pulse generator (1) that is responsible for the episodic secretion of GnRH at the median eminence has yet to be established. It is clear that immortalized GnRH neurons have an intrinsic capacity for the generation of pulsatile neurosecretion, and that GnRH release is highly calcium-dependent and stimulated by cAMP (2–4). A similar process has been shown to be operative in native GnRH neurons (5, 6) and hypothalamic fragments studied *in vitro* (7). Recent observations on GnRH neurons derived from the monkey olfactory placode have shown close synchronization between major Ca^{2+} peaks and episodic GnRH release (8). Although the dependence of GnRH secretion on $[Ca^{2+}]_i$ and its sensitivity to cAMP are well established, relatively little is known about the mechanism(s) that determine the pulsatility of GnRH neuronal secretion. The sparsely distributed GnRH neurons are known to be interconnected (9), and their release of GnRH at the median eminence has been proposed to be synchronized by nitric oxide (10, 11).

In addition to such mechanisms controlling the coordinated activity of the hypothalamic GnRH neurons, the cellular processes that determine their capacity for pulsatile secretion need further definition. The essentiality of both calcium and cAMP in this process has been shown by *in vitro* studies in cultured hypothalamic neurons and GT1 cells. In addition, the expression of GnRH receptors (GnRH-Rs) in GnRH neurons and GT1-7 cells and the ability of GnRH agonists to influence neuropeptide secretion have suggested that autocrine regulation may contribute to the mechanism of episodic GnRH release (6, 12). The coupling of neuronal GnRH-R to multiple G proteins can mediate both activation and inhibition of GnRH release via phospholipase C/inositol trisphosphate ($InsP_3$)/Ca^{2+} and adenylyl cyclase signaling pathways (13), with consequent effects on the GnRH secretory profile. In the present report, the agonist-induced coupling of GnRH-R to individual G proteins was monitored by changes in their membrane-associated α-subunits, specifically $\alpha_{q/11}$, α_s, and α_{i3}. The consequent changes in inositol phosphates, intracellular Ca^{2+} ($[Ca^{2+}]_i$), and cAMP production were analyzed and GnRH secretory profiles were measured in cell perifusion studies to determine the concentration- and time-dependent actions of GnRH agonist and antagonist analogs. These studies have provided further evidence for the role of an autocrine regulatory component in the genesis of pulsatile GnRH secretion.

Methods

Chemicals. [^{125}I]GnRH, [^{3}H]inositol, and Western blotting reagents were obtained from Amersham Pharmacia. [^{125}I]cAMP was from Covance Laboratories (Vienna, VA). GnRH agonist (des-Gly^{10}-[D-Ala^6]GnRH *N*-ethylamide and [D-Ala^6]Ag) and antagonist ([D-*p*Glu^1,D-Phe^2,D-Trp^{3,6}]GnRH and [D-*p*Glu]Antag) analogs were from Peninsula Laboratories. The potent GnRH antagonist analog [Ac-D-Norleucinal(2)^1,D-Phe(*p*Cl)^2,D-3-(3-pyridyl)alanine,D-Citruline^6,D-Ala^{10}] (SB-75) was kindly provided by A. V. Schally (Veterans Affairs Medical Center, New Orleans).

Culture of Hypothalamic and GT1-7 Cells. Hypothalamic tissue removed from 17-day Sprague–Dawley rat fetuses was enzymatically dispersed as described (14). Each dispersed hypothalamus yielded $\approx 1.5 \times 10^6$ cells. Immortalized GnRH neurons (GT1-7 cells) were provided by Richard Weiner (University of California, San Francisco) (15) and cultured under the same conditions as primary hypothalamic cells (12).

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Abbreviations: GnRH, gonadotropin-releasing hormone; GnRH-R, GnRH receptor; $InsP$, $InsP_2$, and $InsP_3$, inositol phosphate, bisphosphate, and trisphosphate, respectively; $[Ca^{2+}]_i$, intracellular Ca^{2+}; Ag, agonist; Antag, antagonist; PTX, pertussis toxin; CTX, cholera toxin.

*On leave from the Department of Pharmacology, Catholic University of the Sacred Heart, 00168 Rome, Italy.

†To whom correspondence should be addressed at: Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Building 49, Room 6A-36, Bethesda, MD 20892-4510. E-mail: catt@helix.nih.gov.

Cell Perifusion Procedure and Hormone Measurement. The bead-attached cells were perifused at a flow rate of 0.15 ml/min at 37°C. Fractions were collected at 5-min intervals and stored at −20°C before RIA. GnRH was measured by using [^{125}I]GnRH (Amersham Pharmacia), GnRH standards (Peninsula Laboratories), and primary antibody donated by V. D. Ramirez (University of Illinois, Urbana). The intra- and interassay coefficients of variation at 50% binding in standard samples (15 pg/ml) were 5% and 7%, respectively. The sensitivity of the assay, defined as twice the standard deviation at zero dose, was 0.2 pg per tube ($n = 6$). There was no detectable cross-reactivity of the GnRH antibody with the GnRH agonist and antagonist analogs at the concentrations used in this study (6).

DNA Constructs and Expression Procedures. cDNAs encoding human rod G protein α transducin ($G_{\alpha t}$), human G protein β1 subunit ($G\beta1$), and human G protein γ2 subunit ($G\gamma2$) cloned into the pcDNA3.1+ expression vector were purchased from the Guthrie cDNA Resource Center (Sayre, PA). GT1-7 cells (10^6) were transfected with pcDNA3.1+ expression plasmids (2 μg) for specific G protein subunits and 6 μl of Lipofectamine 2000 reagent. Control transfections were performed by using the empty vector pcDNA3.1+.

cAMP Production. For studies on cAMP release, GnRH-producing cells were stimulated in serum-free medium (1:1 DMEM/Ham's F-12) containing 0.1% BSA, 30 mg/liter bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. RIA of cAMP was performed as described by using a specific cAMP antiserum at a titer of 1:5,000 (16). The intra-assay coefficient of variation of the assay was 4% at 50% displacement.

Inositol Phosphate Production. Cells were labeled for 24 h in inositol-free DMEM containing 20 μCi/ml [^{3}H]inositol (1 Ci = 37 GBq) as described (17) and then washed with inositol-free M199 medium and stimulated at 37°C in the presence of 10 mM LiCl. The inositol phosphates were fractionated by anion exchange chromatography, and the combined inositol bisphosphate (InsP_2) + InsP_3 fractions eluted with 1 M ammonium formate in 0.1 M formic acid (3 ml per wash) were analyzed by liquid scintillation β-spectrometry.

Calcium Ion Concentration. For single-cell $[Ca^{2+}]_i$ measurements, cultures were incubated at 37°C for 60 min with 2 μM fura-2/acetoxymethyl ester in phenol red-free DMEM. Cells were examined under a ×40 oil-immersion objective during exposure to altering 340- and 380-nm light beams, and the intensity of light emission at 520 nm was measured. All $[Ca^{2+}]_i$ values were derived from a standard curve that was constructed by the addition of known concentrations of Ca^{2+} to 2 μM fura-2/acetoxymethyl ester and measured between 15 and 30 min of exposure to GnRH agonist analog.

Membrane-Associated G_α subunits. Control and agonist-stimulated cells were washed twice with TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.4), scraped from the plates, and lysed by freeze-thawing. After centrifugation at 12,000 × g for 15 min at 4°C, the pellet was resuspended in the TE buffer and stored at −70°C until assayed. Protein contents were measured with a BCA protein assay kit (Pierce). SDS-gel electrophoresis was performed on 12.5% acrylamide gels followed by blotting with poly(vinylidene difluoride) membrane of 0.45-μm pore size. The blots were incubated with primary antibody followed by peroxidase-coupled goat anti-rabbit IgG (H+L) and visualized by chemiluminescence. The immunoreactive bands were analyzed as digitized images by using a GS-700 Imaging Densitometer (Bio-Rad). The optical density (OD) of images is expressed as volume (OD × area) and adjusted for background to give arbitrary units of adjusted volume.

Data Analysis. GnRH pulses were identified and their parameters determined by computerized cluster analysis (18). Individual point standard deviations were calculated by using a power function variance model from the experimental duplicates. A 2 × 2 cluster configuration and a t statistic of 2 for the upstroke and downstroke were used to maintain false-positive and false-negative error rates below 10%. The pulse parameters were analyzed by one-way analysis of variance (ANOVA), and results are expressed as mean ± SEM. Statistical comparisons were performed by using the Kruskal–Wallis test followed by the Mann–Whitney U test.

Results

Coupling of the GnRH-R to $G_{q/11}$ and InsP_3/Ca^{2+} Signaling. As observed previously, cultured GT1-7 neurons exhibited specific, high-affinity binding of the radioiodinated GnRH agonist, des-Gly^{10}[D-Ala^6]GnRH *N*-ethylamide ([^{125}I][D-Ala^6]Ag). The concentration-dependent inhibition of [^{125}I][D-Ala^6]Ag binding by unlabeled GnRH, and GnRH agonist and antagonist analogs, is illustrated in Fig. 1*A*. The estimated IC_{50} values for these ligands were 16 nM for GnRH, 1.0 nM for [D-Ala^6]Ag, and 0.9 nM for the [D-*p*Glu]Antag analog. Treatment of GT1-7 neurons with 1 nM GnRH caused an initial decrease in membrane-bound $\alpha_{q/11}$ subunits after 30 min and a further and significant reduction at 120 min (Fig. 1*B*). Treatment with 1 μM GnRH significantly reduced $\alpha_{q/11}$ after 30 min followed by a return to the control level by 120 min (Fig. 1*C*). In contrast, treatment with the D-*p*Glu GnRH-R antagonist (1 μM) significantly increased $\alpha_{q/11}$ after 120 min of exposure (Fig. 1*D*). The inositol phosphate response (InsP_2 + InsP_3) was dose-dependent from 1 nM to 1 μM GnRH, with an EC_{50} value of 35 nM, and was suppressed by prior addition of the GnRH antagonist (Fig. 1*E*). GnRH-induced activation of neuronal GnRH-Rs caused a dose-dependent increase in $[Ca^{2+}]_i$ with an EC_{50} of 5 nM (Fig. 1*F*). This was prevented by prior treatment with a GnRH antagonist treatment (data not shown).

Agonist stimulation of GT1-7 neurons with 10 nM [D-Ala^6]Ag altered the GnRH secretory profile by increasing peak amplitude (32.2 ± 3.6 vs. 18.2 ± 2.8 pg/ml; $P < 0.01$) and increasing the interpeak interval (58.2 ± 8.6 vs. 33.2 ± 4.8 min; $P < 0.05$; $n = 6$). A representative example of these effects of agonist stimulation is shown in Fig. 1*G*. In contrast to the modulatory action of GnRH agonists on the pattern of neuropeptide secretion (6), treatment with the potent GnRH antagonist, SB-75, abolished episodic neurosecretion and caused a sustained and nonoscillatory increase in GnRH release (Fig. 1*H*). This was followed by a peak of GnRH release during washout of the antagonist and a subsequent return to basal episodic secretion. The mean GnRH level increased from the basal value of 16.7 ± 0.9 to 23.9 ± 1.9 pg/ml ($P < 0.01$; $n = 6$) during the 3-h period of antagonist treatment.

Coupling of GnRH-R to Adenylyl Cyclase Regulatory G Proteins. In hypothalamic cells (Fig. 2*A*) and GT1-7 neurons (Fig. 2*B*), agonist treatment for 30 min stimulated cAMP production at nanomolar GnRH concentrations but had an inhibitory effect at micromolar concentrations. In both cell types, pretreatment with pertussis toxin (PTX, 200 ng/ml, 2 h), which prevents receptor–G protein interaction by catalyzing the ADP ribosylation of α_i subunits, abolished the inhibitory effect of high GnRH levels on cAMP production (Fig. 2 *A* and *B*). Treatment of GT1-7 cells with 1 nM GnRH reduced membrane-associated α_{i3} at 30 min and caused a further decrease at 120 min (Fig. 2*C*). However, treatment with 1 μM GnRH significantly reduced α_{i3} after 30 min followed by a return to the control level after 120 min (Fig. 2*D*). In contrast, membrane-associated α_{i3} was increased significantly during treatment with a GnRH-R antagonist (Fig. 2*E*). Exposure of GT1-7 neurons with PTX (200 ng/ml) also caused a significant increase in membrane-associated α_{i3}. In contrast, cholera toxin (CTX)-induced activation of adenylyl cyclase by ADP-ribosylation of G_s had no effect on α_{i3} (Fig. 2*F*). In perifusion studies, treatment with PTX abolished

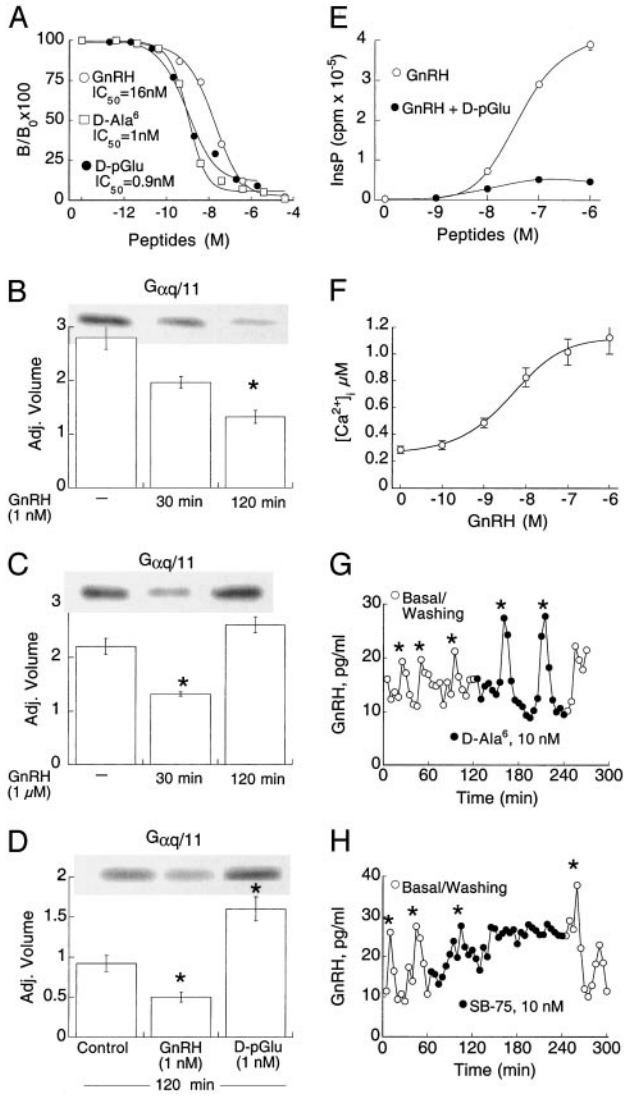

Fig. 1. Coupling of the GnRH-R to phospholipase C/InsP_3/Ca^{2+} signaling and episodic GnRH release in GT1-7 cells. (*A*) Binding properties of GnRH, the D-Ala^6 agonist analog, and the D-*p*Glu antagonist analog at the neuronal GnRH-R. (*B–D*) Time- and dose-dependent effects of GnRH and the D-*p*Glu antagonist analog on membrane-associated $\alpha_{q/11}$ subunits. (*E* and *F*) Dose-dependent actions of GnRH on InsP_3 production and $[Ca^{2+}]_i$ levels. (*G* and *H*) Modulation of GnRH pulse frequency and amplitude by the D-Ala^6 agonist analog and the SB-75 GnRH antagonist (filled circles). Basal/washing represents GnRH profile during perifusion with medium alone and washout of the treatment. Asterisks indicate significant differences ($P < 0.05$) compared with the controls.

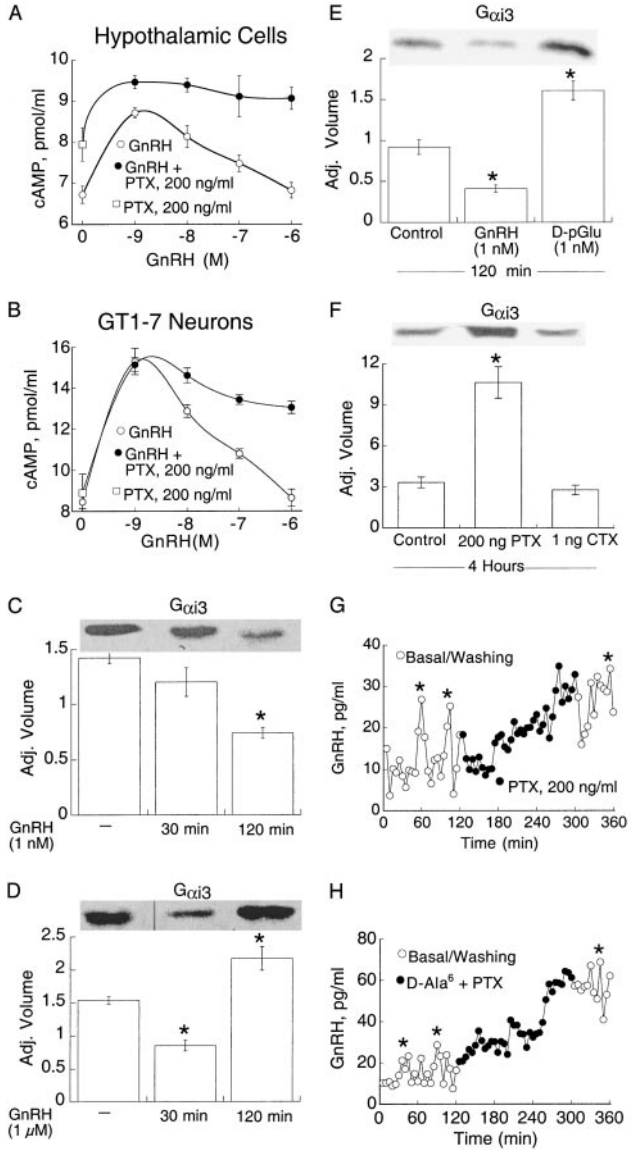

Fig. 2. Coupling of the GnRH-R to adenylyl cyclase and regulatory G proteins in GT1-7 cells. (*A* and *B*) GnRH-induced biphasic changes in cAMP production (open circles) with stimulation at low agonist concentrations and progressive inhibition at higher concentrations in cultured hypothalamic cells (*A*) and GT1-7 neurons (*B*). In the presence of PTX (filled circles), the inhibition of cAMP production by high GnRH concentrations is diminished. (*C* and *D*) Dose- and time-dependent effects of GnRH on membrane-associated α_{i3} immunoreactivity. (*E*) PTX and CTX on membrane-associated α_{i3} immunoreactivity. (*F*) Effects of a GnRH antagonist (D-*p*Glu) on membrane-associated $\alpha_{q/11}$. (*G* and *H*) Effects of PTX alone (*G*) or with a GnRH agonist (D-Ala^6) (*H*) on episodic GnRH release. Basal/washing indicates the GnRH profile during perifusion with medium alone and/or washout of the treatment. Asterisks indicate significant differences ($P < 0.05$) compared with the control.

pulsatile neurosecretion and caused a monotonic rise in GnRH release (Fig. 2*G*) due to increased cAMP production. PTX treatment also prevented the GnRH-induced modulation GnRH pulse frequency and amplitude (Fig. 2*H*). Transient expression of G protein $\beta_1\gamma_2$ subunits in GT1-7 neurons to bind free α subunits reduced basal cAMP production and abolished the cAMP response to nanomolar GnRH concentrations (Fig. 3*A*). In contrast, transient expression of the transducin α-subunit, a scavenger of $\beta\gamma$ subunits, abolished the G_i-mediated inhibitory effect of high GnRH concentrations on cAMP production (Fig. 3*B*).

More detailed time-course studies showed that nanomolar concentrations of GnRH stimulated cAMP production to a maximal level after 30 min followed by a decline to the basal level at 60 min. In contrast, the effect of 1 μM GnRH on cAMP production was solely inhibitory (Fig. 4*A*). Activation of GnRH-Rs also influenced α_s subunit levels, which were reduced during treatment with nanomolar concentrations of GnRH that elevated cAMP production. However, at high GnRH concentrations that caused a PTX-sensitive decrease in cAMP production, there was no significant change in α_s subunits at 30 min (Fig. 4*B*). Treatment of GT1-7 cells with 8-bromo-cAMP potentiated GnRH secretion, in part by increasing the basal level of GnRH release, but had little effect on peak frequency (Fig. 4*C*). CTX-treated GT1-7 cells also showed prominent increases in cAMP production (Fig. 4*D*) that paralleled the changes caused by nanomolar concentrations of GnRH and the

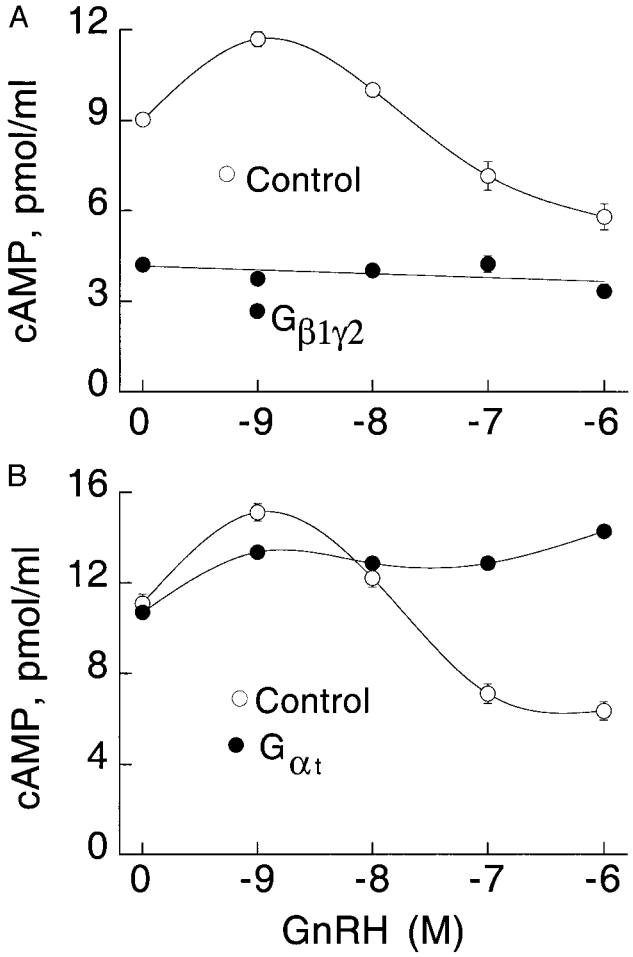

Fig. 3. Modulation of cAMP production in GT1-7 cells by G protein $\beta\gamma$ subunits. (*A*) Abolition of the stimulatory actions of GnRH on cAMP production by transient transfection with cDNA encoding $\beta_1\gamma_2$ subunits. (*B*) Prevention of the inhibition of cAMP production at high GnRH concentrations by transient expression of the $\beta\gamma$ subunit sequestrant, $G_{\alpha t}$.

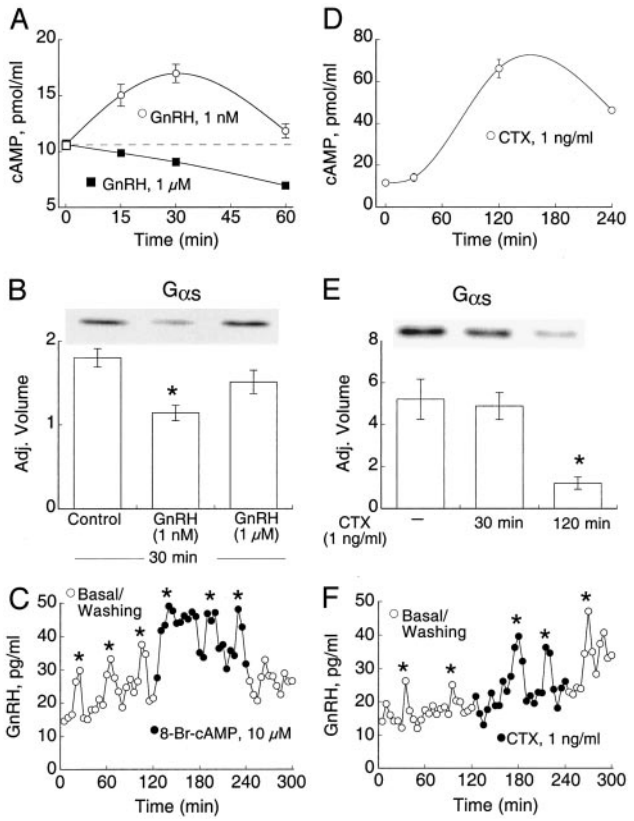

Fig. 4. Coupling of GnRH-R to adenylyl cyclase stimulatory G protein in GT1-7 cells. (*A*) Time-dependent actions of low (open circles) and high (filled squares) GnRH concentrations on cAMP production. (*B*) Effects of GnRH treatment on membrane-associated α_s. (*C*) Effects of 8-bromo-cAMP on pulsatile GnRH release. (*D*) Effects of choleragen on cAMP production. (*E*) CTX-induced changes in membrane-associated α_s. (*F*) Effect of CTX on pulsatile GnRH release. Asterisks indicate significant differences ($P < 0.05$) compared with the control.

concomitant decreases in α_s (Fig. 4*E*). In perifused GT1-7 cells, CTX treatment enhanced GnRH peak amplitude as a consequence of the marked increase in cAMP production (Fig. 4*F*).

Differential Regulation of GnRH-R Signaling Pathways. GnRH-induced inositol phosphate (Ins*P*) production in GT1-7 cells showed a monophasic increase to a plateau level after 60 min of incubation with 100 nM GnRH (Fig. 5*A*). Low GnRH concentrations (1 nM) likewise caused a monophasic increase in Ins*P* production that reached ≈2% of that elicited by 100 nM GnRH (data not shown). In contrast, GnRH-induced cAMP production was differentially regulated by low and high GnRH concentrations. As before, treatment with 1 nM GnRH for 30 min caused a biphasic cAMP response with maximal response after 30 min and a decline to the basal level after 60 min (Fig. 5*A*). However, 1 μM GnRH only reduced cAMP production, with a maximal inhibitory effect after 60 min (Fig. 5*A*).

The Ins*P* and cAMP responses were also differentially regulated during agonist concentration-dependence studies. Whereas maximal Ins*P* production was achieved with high GnRH concentrations, cAMP production was minimal at such agonist levels. In contrast, maximal cAMP production occurred during stimulation with low GnRH concentrations, at which Ins*P* production was minimal (Fig. 5*B*). These time- and dose-dependent differences in the activation of specific second messengers were also reflected in the profile of GnRH release. In perifused GT1-7 neurons, the GnRH secretory profile was characterized by clearly defined peaks with a mean amplitude of 15.4 ± 2.8 pg/ml ($n = 3$) and interpeak intervals of 37.4 ± 5.3 min (Fig. 5*C*). Application of 10 nM [D-Ala^6]Ag increased both GnRH peak amplitude (29.3 ± 4.5 pg/ml; $n = 3$; $P < 0.05$) and the interpeak interval (56.4 ± 8.3 min; $P < 0.05$) (Fig. 5*D*). The transient peaks that appear to be induced by activation of the Ins*P*/Ca^{2+} signaling pathway (Fig. 5 *C* and *D*, filled squares) and neuronal firing may be terminated by a time- and concentration-dependent switch of coupling from G_s to adenylyl cyclase inhibitory G proteins (Fig. 5 *C* and *D*, filled circles). The initial increase and sustained basal GnRH release could be a consequence of GnRH-R coupling to G_s and increased cAMP production (Fig. 5 *C* and *D*, open circles), as indicated in Fig. 6.

Discussion

The episodic secretory activity of GnRH neurons is essential for the maintenance of normal gonadotropin secretory profiles and gonadal function and is influenced by neuronal inputs, neuropeptides, and gonadal hormones that modify the pulsatile mode of GnRH release from the median eminence (19). Stimulation of the endogenous GnRH-R expressed in GT1-7 neurons by GnRH and its agonist analogs activates three G proteins, as indicated by the time- and dose-dependent release of their specific α-subunits from the plasma membrane (20). Exposure of GT1-7 neurons to low nanomolar GnRH concentrations reduced membrane-associated $\alpha_{q/11}$ subunits and increased inositol phosphate production and $[Ca^{2+}]_i$. In contrast, micromolar agonist

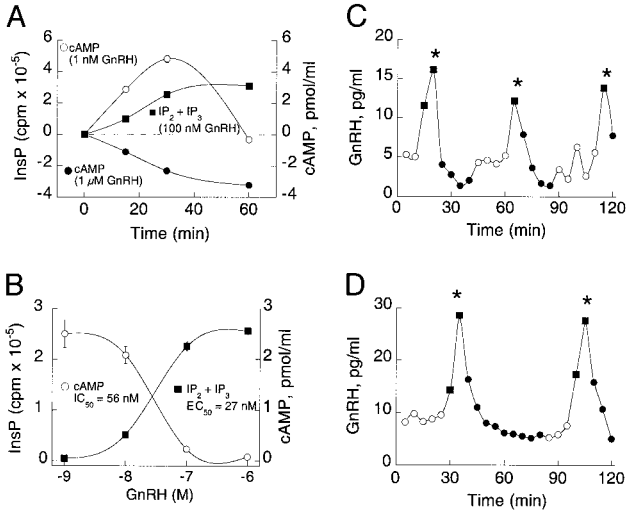

Fig. 5. Differential regulation of signaling pathways and GnRH release in cultured GT1-7 neurons. (*A*) Time-dependent effects of low (open circles) and high (filled circles) GnRH concentrations on cAMP production and InsP_3 accumulation (filled squares). (*B*) Reciprocal dose-dependent effects of GnRH on cAMP production and Ins*P* accumulation. (*C*) Basal pulsatile GnRH release in perifused cells. (*D*) Effects of [D-Ala^6]Ag on GnRH pulse amplitude and frequency. The symbols shown in *C* and *D* indicate the phases at which episodic secretion is proposed to be governed by activation of the InsP_3/Ca^{2+} and cAMP signaling pathways (filled squares) and G_i-mediated inhibition of secretion (filled circles). The intervening period is shown by open circles.

concentrations caused a rapid decrease in membrane-associated $\alpha_{q/11}$ that returned to the control level during sustained treatment (20). This was associated with maximal inositol phosphate production and $[Ca^{2+}]_i$ mobilization as well as prominent increases in GnRH peak amplitude and prolongation of the interpulse interval (12). These findings indicate that GnRH-induced activation of $\alpha_{q/11}$ causes stimulation of phospholipase C/InsP_3/Ca^{2+} signaling that in turn influences pulsatile GnRH secretion. The converse observation, of increased membrane-associated $\alpha_{q/11}$ during GnRH antagonist treatment, suggests that tonic autocrine stimulation of the GnRH-R is associated with partial activation of $G_{q/11}$ in GnRH neuronal cells.

Activation of GnRH-Rs in GnRH neurons and GT1-7 cells also affected adenylyl cyclase activity in a biphasic manner such that cAMP production was stimulated at nanomolar agonist levels and

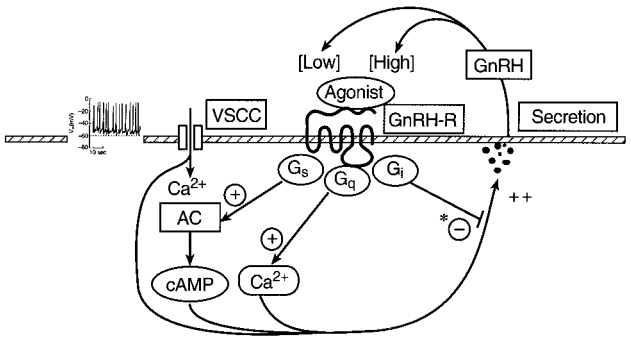

Fig. 6. A proposed mechanism of pulsatile GnRH secretion. GnRH neuronal firing of Ca^{2+}-dependent action potentials promotes Ca^{2+} influx, stimulation of cAMP production, and Ca^{2+}/protein kinase C signaling, all of which elevate GnRH release. The autocrine switch from G_s to G_i at high local GnRH concentrations interrupts the rise in neurosecretion and is followed by a fall to baseline and subsequent reactivation of secretion via the resurgent Ca^{2+}/cAMP signaling pathways. VSCC, voltage-sensitive calcium channels; AC, adenylyl cyclase.

decreased by micromolar concentrations. The dependence of the former effect on activation of G_s was indicated by the marked inhibition of basal and GnRH-induced cAMP production after overexpression of $G\beta_1\gamma_2$ subunits in GT1-7 neurons. The ability of the neuronal GnRH-R to also interact with G_i proteins was indicated by the manner in which PTX pretreatment abolished the inhibitory effect of high GnRH levels on cAMP formation. This was supported by the finding that overexpression of transducin α-subunits in GT1-7 cells to bind free $\beta\gamma$ subunits prevented the inhibitory actions of high GnRH concentrations. These observations are consistent with the ability of PTX to abrogate the inhibitory actions of high GnRH concentrations on cAMP production by inactivating G_i.

The time- and dose-dependent decreases in membrane-bound α_{i3} during GnRH treatment and the increases therein during GnRH antagonist analog and PTX treatment reflect the coupling of activated GnRH-Rs to inhibitory G_i protein(s) in GnRH neurons. Furthermore, the monotonic increase and cessation of pulsatile GnRH release and loss of agonist-induced modulation of pulse frequency and amplitude in perifused GT1-7 neurons during PTX treatment indicate that such inhibitory coupling of the GnRH-R is required for the maintenance of pulsatile GnRH release. In addition, treatment of GT1-7 neurons with high GnRH concentrations or with gonadotropins (luteinizing hormone/human chorionic gonadotropin) inhibits adenylyl cyclase and cAMP production via receptor-dependent activation of G_i (13). Such G_i-mediated inhibition of GnRH release, with a decrease in membrane-associated α_{i3}, and PTX-sensitive inhibition of cAMP production also occurs in GT1-7 neurons during agonist activation of M2 muscarinic receptors (21).

The switch in coupling of neuronal GnRH-Rs from G_s to G_i at high agonist concentrations provides a mechanism through which their activation by rising endogenous GnRH levels could terminate the initial stimulatory action of GnRH on its own production through G_q-mediated signals and cAMP. Such a dynamic recycling of G_i in the genesis of GnRH-R-driven episodic neurosecretion is consistent with the large increase in membrane-associated α_{i3} subunits that occurs during both PTX treatment and antagonist blockade of the GnRH-R. PTX inactivates the G_i heterotrimer, and the GnRH antagonist arrests its activation by rising agonist levels, in both cases preventing dissociation into its active α_i and $\beta\gamma$ subunits. In addition to its impairment of cAMP production, the inhibitory effect of G_i activation by GnRH, luteinizing hormone/hCG, and M2 agonists on pulsatile GnRH secretion could result from the opening of G protein-gated inwardly rectifying potassium (GIRK) channels. Such a mechanism is analogous to the manner in which GIRK channels mediate the inhibitory action of acetylcholine via M2 muscarinic receptors in the cardiac conduction system (22).

GnRH-induced coupling to multiple G proteins has been observed in pituitary-derived GGH_3 cells expressing GnRH-R and transiently transfected with cDNAs for $\alpha_{q/11}$, α_{i2}, and α_s (23). Activation of the GnRH-R in LβT2 gonadotrope cells also stimulates G_q and G_s signaling to regulate gene expression (24), and the inhibitory action of GnRH in endometrial tumor cells is associated with ADP ribosylation of membrane-bound G_i (25). Stimulation of cAMP production by nanomolar GnRH concentrations was associated with a decrease in membrane-bound α_s subunits, consistent with receptor-induced G_s dissociation and α-subunit redistribution during GnRH-R activation. In COS-7 cells transiently expressing the GnRH-R, agonist-stimulated cAMP production depends on specific amino acids in the first intracellular loop that are not essential for activation of the phosphoinositide signaling pathway (26). Stimulation of cAMP production by GnRH was also observed in COS-7 cells transfected with the GnRH-R and Ca^{2+}-dependent adenylyl cyclase type I (AC I) and was proposed to be exclusively due to increased cytosolic Ca^{2+} during GnRH treatment (27). Immortalized

GnRH neurons express the endogenous GnRH-R as well as AC I, and the stimulatory actions of GnRH on cAMP production are mediated by both Ca^{2+} entry through L-type channels and GnRH-R coupling to G_s (13).

In GT1-7 neurons, membrane-associated α_s was predictably decreased during treatment with CTX, with a concomitant increase in cAMP production. However, treatment with micromolar GnRH concentrations caused no detectable change in α_s after 30 min, consistent with a rapid switch in the coupling of GnRH-R from G_s to G_i. Treatment of perifused GT1-7 neurons with 8-bromo-cAMP and CTX also caused sustained increases in basal GnRH release and amplified GnRH pulse amplitude. Our findings in immortalized GnRH neurons indicate that low GnRH concentrations promote the coupling of GnRH-R to G_s, stimulate cAMP production, and increase GnRH release. The G_s coupling initiated by low GnRH concentrations, and the consequent increase in cAMP production, in conjunction with activation of $G_{q/11}$-dependent calcium signaling maintain basal and transient increases in GnRH release. In contrast, the time- and dose-dependent increase in coupling to G_{i3} inhibits GnRH release and transiently interrupts receptor signaling. Thus, the time- and dose-dependent switching of GnRH-R coupling between specific G proteins could dynamically regulate neurosecretion and maintain the episodic GnRH release that is characteristic of GnRH neuronal function *in vivo* and *in vitro*.

The generation of episodic neurosecretion by the GnRH neuron has been ascribed to the effects of intermittent bursts of action potentials and calcium oscillations (8, 28) and also to an inhibitory autocrine feedback action of GnRH on its own secretion (6, 12). Such a negative regulatory action of GnRH on its neuronal cells of origin presumably depends on peptidergic synaptic interconnections within the GnRH neuronal network. Neurotransmission by GnRH was first observed in bullfrog sympathetic ganglia (29) and subsequently in rat hippocampal neurons (30) and is believed to mediate neuroendocrine and sexual behavioral responses in the mammalian brain. Cultured GT1-7 cells, similar to normal GnRH neurons, exhibit sparse synaptic connections between individual cells that could serve to coordinate the activity of the neuronal network (31). These cells form very few gap junctions under the culture conditions of the present study and are predominantly possible by fibers that terminate on the perikarya and axons of adjacent neurons.

The stimulatory action of cAMP on GnRH release does not depend on activation of protein kinase A but has been attributed to the opening of cyclic nucleotide-gated cation channels in the plasma membrane of normal and immortalized GnRH neurons (32, 33). This increases cell excitability and enhances firing of action potentials, leading to depolarization and elevation of $[Ca^{2+}]_i$. The exact manner in which cAMP contributes to the episodic mode of GnRH secretion has yet to be determined, but its importance in the control of GnRH release is indicated by a recent study in rats expressing an active phosphodiesterase transgene in their GnRH neurons (34). However, it is likely that cAMP is essential but not alone sufficient for the generation of pulsatile GnRH release.

Several other plausible mechanisms for the generation of pulsatile GnRH release have been suggested. These include the spontaneous electrical activity of single GnRH neurons (35, 36), bursts of action potentials as in other neuroendocrine cells (37–39), and the intrinsic activity of the GnRH neuronal network (40), with independent, coupled, and triggered modes of synchronized firing and neurosecretion (41). The mechanism described in the present report incorporates two additional factors: the essential role of negative GnRH feedback, as revealed by the abolition of pulses during antagonist blockade, and the marked inhibition of episodic neurosecretion by activation of G_i. The coexistence of multiple regulatory mechanisms could provide a degree of redundancy in the maintenance of such a crucial component of the reproductive process. It is also conceivable that this multifactorial system could reflect the gradation from simple to more complex neuroendocrine control systems for regulating hypothalamo-pituitary function and gonadal activity during the evolution of the GnRH pulse generator.

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